

5-HT-Induced, 5-HT₃ Receptor-Mediated, and Ruthenium Red- and Capsaicin-Sensitive Positive Chronotropic Effects in the Isolated Guinea Pig Atrium

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ABSTRACT—We investigated the mechanisms of 5-HT-induced tachycardia, which we reported previously to be triggered by 5-HT₃ receptor stimulation, in the isolated guinea pig atrium in comparison with that induced by isoproterenol and histamine. We found that 5-HT-induced tachycardia was completely inhibited by ruthenium red. 5-HT-induced tachycardia was reduced in the capsaicin pre-treated atrium as well as in the presence of capsaicin. The effects of isoproterenol and histamine were not affected by ruthenium red or capsaicin treatment. Furthermore, 5-HT-induced tachycardia was found to be potentiated by thiorphan, an inhibitor of peptide degradation. Calcitonin gene-related peptide (CGRP) (1–37), a full agonist of CGRP₁-like receptors, was found to act selectively as a potent stimulator of chronotropic action. CGRP (8–37), an antagonist of CGRP₁-type receptors, inhibited 5-HT-induced tachycardia as well as effects induced by CGRP (1–37). The observation that tetrodotoxin failed to affect 5-HT-induced tachycardia excluded the involvement of 5-hydroxytryptaminergic interneurons. Thus, we confirmed that the mechanism of 5-HT-induced tachycardia is distinct from that induced by isoproterenol and histamine. In conclusion, the activation of 5-HT₃ receptors on the sensory nerve terminals brought about ruthenium red-sensitive Ca²⁺ influx and resulted in the release of CGRP from capsaicin-sensitive stores, and then CGRP stimulated CGRP₁-like receptors to produce 5-HT-induced tachycardia.

Keywords: Serotonin, Guinea pig atrium, Tachycardia, 5-HT₃ receptor, Calcitonin gene-related peptide

Serotonin (5-hydroxytryptamine, 5-HT) is one of the biogenic amines that mediate the transmission of a wide variety of neuronal and non-neuronal information and play important roles in peripheral tissues as well as in the central nervous system. Peripherally, 5-HT is synthesized in enterochromaffin cells and taken up by blood platelets, which distribute it throughout the whole body via the vascular system. 5-HT released from platelets in response to various kinds of stimulation exerts an effect on the cardiovascular system including the heart. Therefore, it would be expected that these systems might emerge in some pathological states such as ischemic heart disease or pulmonary hypertension. Further elucidation of mechanism of regulation of cardiovascular function by 5-HT will clarify its physiological and pathological roles.

5-HT-induced tachycardia in the guinea pig atrium is

mediated by mechanisms different from those active in other species and employs different receptor subtypes (1, 2). For example, the effects of 5-HT in the human and porcine heart were suggested to be mediated by direct activation of the 5-HT₄ receptor subtype coupled to activation of adenylate cyclase (3). In rat heart preparations, the 5-HT₂ receptor subtype has been suggested to be involved in the effect of 5-HT (4). In the guinea pig heart, it was initially reported that the action of 5-HT is tyramine-like and was blocked by a β -adrenoceptor antagonist (5). In our previous study, however, we concluded that 5-HT can produce positive chronotropic effects that are not due to β -adrenergic stimulation, but are mediated by activation of 5-HT₃ receptors (6). Furthermore, we demonstrated that ethanol inhibits 5-HT-induced tachycardia but not isoproterenol- and histamine-induced tachycardia in the isolated guinea pig atrium (7). Thus, in the guinea pig atrium 5-HT₃ receptors have been suggested to function as ligand-gated ion channels with selective sensitivity to alcohol, similarly to other

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ligand-gated ion channels such as GABA_A (8), nicotinic (9) and NMDA receptors (10). We proposed the use of 5-HT-induced tachycardia in the isolated guinea pig atrium as a simple and potentially useful experimental tool for investigating 5-HT₃ receptor-mediated cellular responses as well as its functions. There is evidence suggesting the production of various second messengers, such as nitric oxide and c-GMP, upon activation of 5-HT₃ receptors (11, 12), but the relevance of these observations remains to be clarified. In this respect, we have been studying the mechanism of 5-HT-induced tachycardia induced by 5-HT₃ receptor activation in the isolated guinea pig atrium.

In the present study, we elucidated the mechanisms of 5-HT-induced tachycardia by comparison with that induced by isoproterenol and by histamine; the latter is mediated by activation of Gs protein-coupled receptors followed by elevation of intracellular cAMP levels. The mechanism of 5-HT-induced tachycardia was confirmed to be distinct from the effects of isoproterenol and histamine. We also found that 5-HT-induced tachycardia in the isolated guinea pig atrium is mediated by release of cardioaccelerator peptide, which is sensitive to ruthenium red and capsaicin, but insensitive to tetrodotoxin. Our results suggested that calcitonin gene related peptide (CGRP) is a good candidate for the cardioaccelerator peptide.

MATERIALS AND METHODS

Experiments on spontaneously beating atria

Procedures involving animals and their care were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society. The paired atria were dissected out from the hearts of freshly killed guinea pigs and suspended in Ringer-Locke solution (composition: 9.0 g/l NaCl, 0.42 g/l KCl, 0.24 g/l CaCl₂, 1.0 g/l glucose, 0.2 g/l NaHCO₃) gassed with 95% O₂ and 5% CO₂ at 30°C. The atrium was connected under a resting tension of 0.5 g to an isometric force transducer connected to an amplifier (EF-601G; Nihon Kohden, Tokyo) and a heart rate counter (AT-601G, Nihon Kohden). Contractile strength and heart rate were recorded continuously on a microcomputer system equipped with an A/D converter. Each atrium was treated with agonist (5-HT in the presence of 10 µM atropine, isoproterenol or histamine) in a cumulative manner (control experiment), and then the effects of drugs were estimated by addition of drugs 5 min before the second agonist stimulation. The agonists were applied at 1-h intervals. Positive chronotropic effects (tachycardia) of the agonists are expressed as percentages of the maximal increase in heart rate caused by the agonists in each control experiment. The maximum response obtained with 5-HT was approximately 45% and 40% of those with isoprot-

erenol and histamine, respectively.

Statistical analyses

Results are expressed as means ± S.E.M., and the statistical significance of differences was determined by one-way analysis of variance followed by Dunnett's test.

Chemicals

The following reagents were obtained from the indicated sources: 5-HT (5-hydroxytryptamine creatinine sulfate) was from E. Merck (Darmstadt, Germany); histamine 2HCl, (-)-isoproterenol HCl, capsaicin, thiorphan and tetrodotoxin were from Sigma Chemical Co. (St. Louis, MO, USA); ruthenium red was from Chroma-Gesellschaft Schmid GmbH & Co. (Münster, Germany); CGRP (1–37), CGRP (8–37) and adrenomedullin (25–52) were from Peptide Institute, Inc. (Osaka). All other chemicals used in this study were of analytical grade and were obtained from commercial sources.

RESULTS

Effects of ruthenium red on 5-HT-, isoproterenol- and histamine-induced tachycardia

The effects of ruthenium red on 5-HT-, isoproterenol- and histamine-induced positive chronotropic actions in the isolated guinea pig atrium were examined. As shown in Fig. 1, 5-HT-induced positive chronotropic effects were completely inhibited by 5 µM ruthenium red. On the other hand, neither isoproterenol- nor histamine-induced effects were affected by the same concentration of ruthenium red (Fig. 1).

5-HT- and capsaicin-induced tachycardia

Capsaicin at 10 µM produced positive chronotropic action at the first application to the isolated guinea pig atrium. The effect of capsaicin was transient, and the maximal effect was $164.0 \pm 18.7\%$ ($n=5$) as compared with that of 10 µM 5-HT as 100% (Fig. 2). The elevated heart rate induced by capsaicin was recovered to the control level 6 to 9 min after application. The second application of capsaicin after washing with Ringer-Locke solution did not cause any positive chronotropic effects, while the second application of 5-HT showed the same effect as its first application (Fig. 2).

Effects of capsaicin on 5-HT-, isoproterenol- and histamine-induced tachycardia

5-HT, isoproterenol or histamine was applied to the capsaicin-treated atrium preparations. When applied with capsaicin, the effect of 5-HT was markedly reduced in comparison to the first application (Fig. 3). On the other hand, when isoproterenol or histamine was applied, they

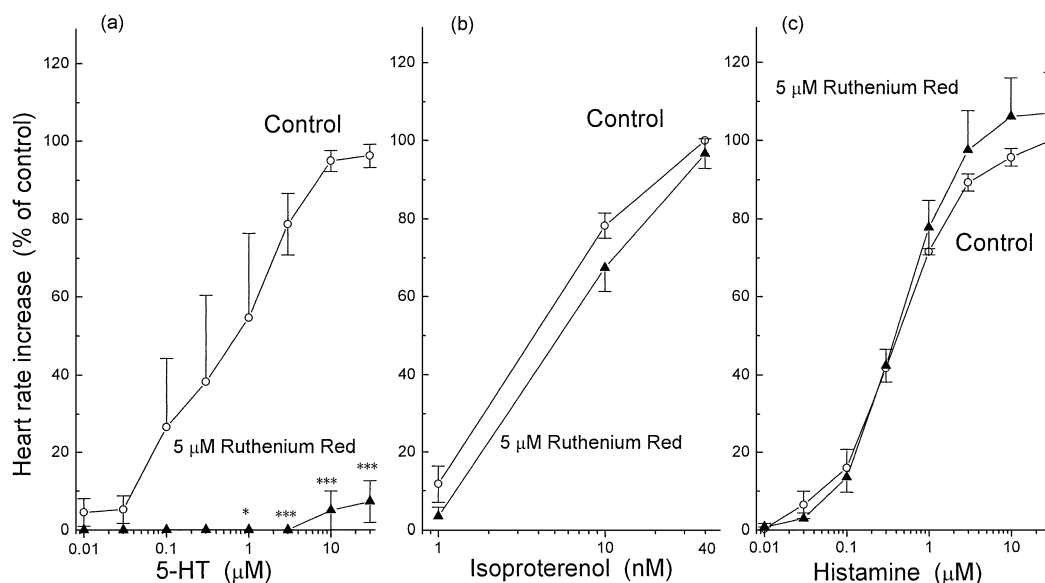


Fig. 1. Concentration-effect curves for 5-HT (a), isoproterenol (b) and histamine (c) in the absence (control) and presence of ruthenium red ($5 \mu\text{M}$) in the isolated guinea pig atrium. Chronotropic effects of 5-HT, isoproterenol or histamine are expressed as percentages of the maximal increase in heart rate caused by the agonists in each control experiment. Each point represents the mean \pm S.E.M. of three to four independent experiments. Significance: $*P < 0.05$, $***P < 0.001$ (vs control).

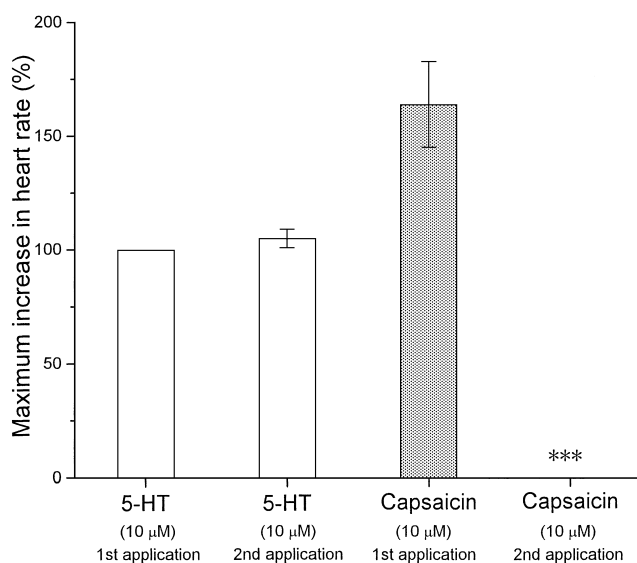


Fig. 2. 5-HT- and capsaicin-induced positive chronotropic effects in the isolated guinea pig atrium. Chronotropic effects of 5-HT ($10 \mu\text{M}$) or capsaicin ($10 \mu\text{M}$) are expressed as percentages of the maximal increase in heart rate caused by first application of 5-HT in each experiments. Each point represents the mean \pm S.E.M. of three to four independent experiments. Significance: $***P < 0.001$ (vs 1st application).

produced the same positive chronotropic effects as their first application (Fig. 3). Moreover, we analyzed the effects of these cardio-stimulant drugs on capsaicin-treated atrium preparations that had been washed with Ringer-Locke solution. While the positive chronotropic effects induced

by isoproterenol and histamine did not change in the capsaicin pre-treated atrium, the effect of 5-HT was significantly reduced in comparison to the first application (Fig. 3).

Effects of peptidase inhibitor on 5-HT-induced tachycardia and several bioactive peptides on rate of rhythm in the isolated guinea pig atrium

We found that the 5-HT-induced positive chronotropic effect was significantly potentiated in the presence of thiorphan, a peptidase inhibitor (Fig. 4), indicating the possible involvement of bioactive peptides in the effect of 5-HT. Then, we analyzed the effects of several bioactive peptides that were confirmed immunohistochemically to be present in the guinea pig atrium. Among the peptides tested, bath application of substance P, somatostatin, vasoactive intestinal peptide (VIP) and neuropeptide Y at concentrations up to 100 nM did not cause significant changes in the heart rate, and endothelin showed a negative chronotropic effect (data not shown). CGRP (1–37), a full agonist of CGRP_1 receptors, revealed dose-dependent positive chronotropic effects at nanomolar concentrations (Fig. 5). The maximal effect induced by 100 nM CGRP (1–37) was more than 200% of that induced by 5-HT (Fig. 5).

Effects of CGRP_1 -like receptor antagonist and related substances on 5-HT-induced tachycardia

The effects of CGRP_1 -like receptor antagonists on 5-HT-induced positive chronotropic action were examined. CGRP (8–37), a ligand that preferentially antagonizes the

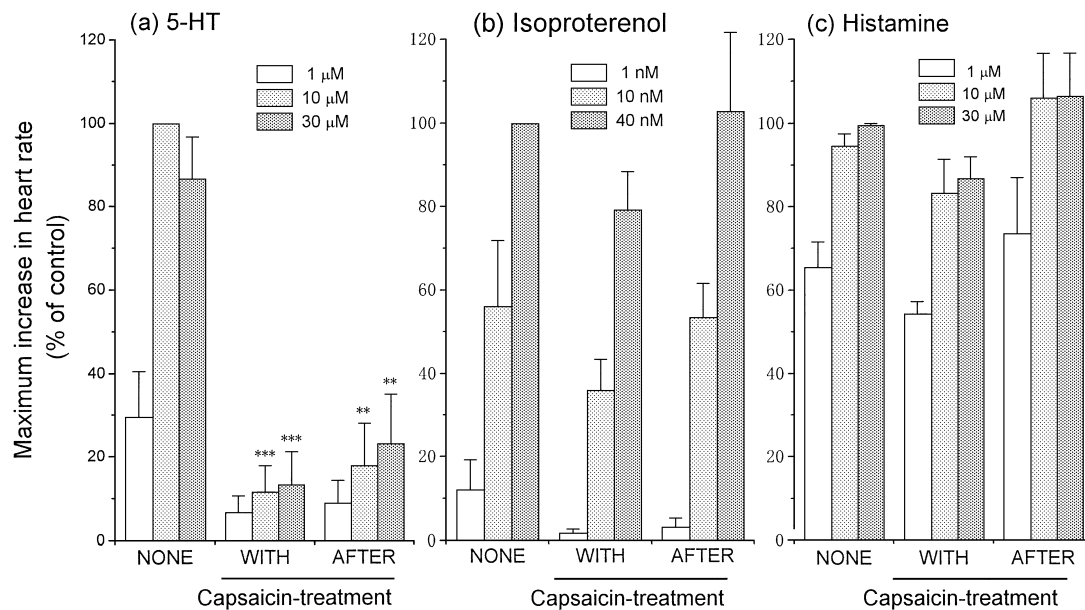


Fig. 3. Concentration-effect curves for 5-HT (a), isoproterenol (b) and histamine (c) in the absence (none), with 10 μ M capsaicin treatment (with) and after the capsaicin treatment (after) in the isolated guinea pig atrium. Chronotropic effects of 5-HT, isoproterenol or histamine are expressed as percentages of the maximal increase in heart rate caused by the agonists in each control experiment. Each point represents the mean \pm S.E.M. of three to four independent experiments. Significance: ** P <0.01, *** P <0.001 (vs none).

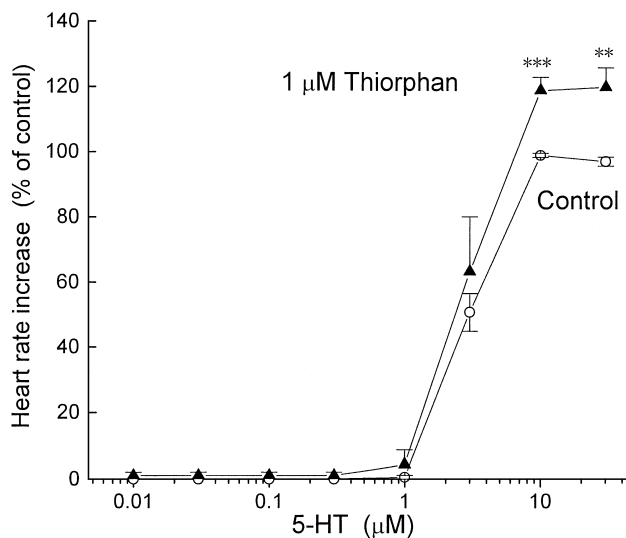


Fig. 4. Concentration-effect curves for 5-HT in the absence (control) and presence of thiorphan (1 μ M) in the isolated guinea pig atrium. Chronotropic effects of 5-HT are expressed as percentage of the maximal increase in heart rate caused by 5-HT in each control experiment. Each point represents the mean \pm S.E.M. of three independent experiments. Significance: ** P <0.01, *** P <0.001 (vs control).

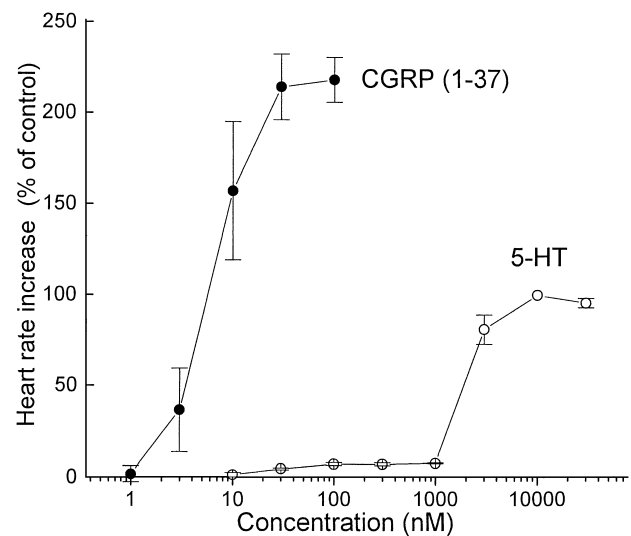


Fig. 5. Concentration-effect curves for 5-HT and CGRP (1–37) in the isolated guinea pig atrium. Chronotropic effects of 5-HT or CGRP (1–37) are expressed as percentages of the maximal increase in heart rate caused by 5-HT in each control experiment. Each point represents the mean \pm S.E.M. of three independent experiments.

CGRP₁-like receptor subtype, significantly inhibited the positive chronotropic effect of 5-HT (Fig. 6) to the same extent as the effects of CGRP (1–37) (data not shown). Adrenomedullin (22–52), an antagonist of the CGRP-

related peptide adrenomedullin, did not inhibit the effects of 5-HT (Fig. 6).

Effects of tetrodotoxin on 5-HT-induced tachycardia

To determine the involvement of activation of inter-

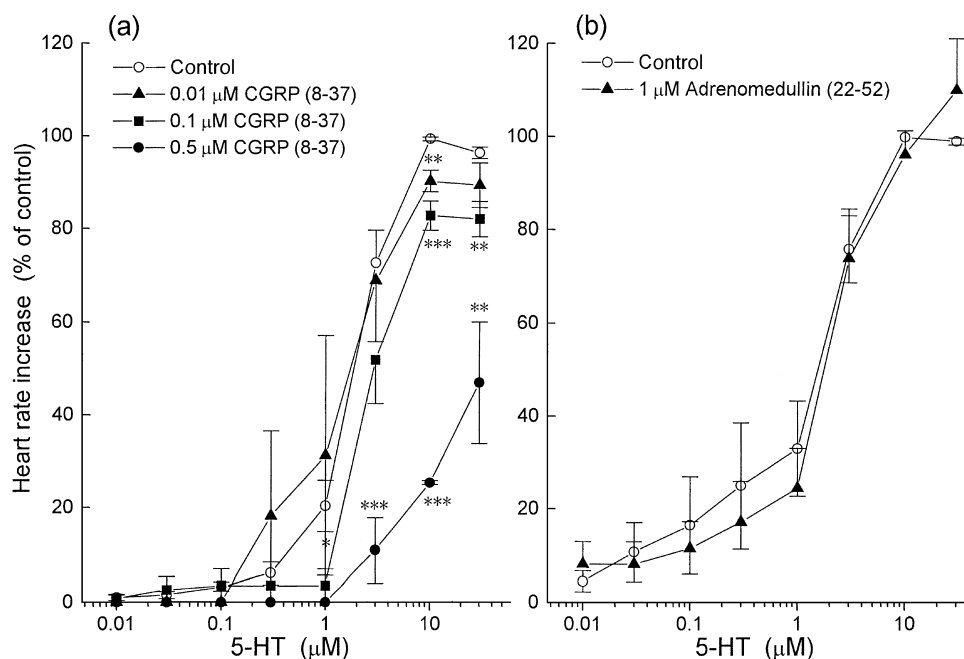


Fig. 6. Concentration-effect curves for 5-HT in the absence (control) and presence of CGRP (8–37) (0.01–0.5 μ M) (a) and adrenomedullin (22–52) (1 μ M) (b) in the isolated guinea pig atrium. Chronotropic effects of 5-HT are expressed as percentages of the maximal increase in heart rate caused by 5-HT in each control experiment. Each point represents the mean \pm S.E.M. of three independent experiments. Significance: * P <0.05, ** P <0.01, *** P <0.001 (vs control).

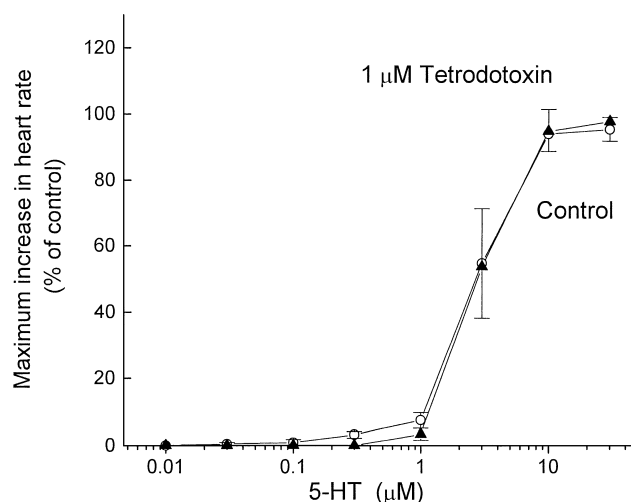


Fig. 7. Concentration-effect curves for 5-HT in the absence (control) and presence of tetrodotoxin (1 μ M) in the isolated guinea pig atrium. Chronotropic effects of 5-HT are expressed as percentages of the maximal increase in heart rate caused by 5-HT in each control experiment. Each point represents the mean \pm S.E.M. of three independent experiments.

neurons in the effects of 5-HT, we analyzed the influence of tetrodotoxin, a specific channel blocker of voltage-gated Na^+ channels. Tetrodotoxin was found to have no effect on 5-HT-induced tachycardia (Fig. 7).

DISCUSSION

Ruthenium red has been established as an important blocker of the Ca^{2+} influx and the Ca^{2+} -dependent release of peptides in several nervous tissue preparations. These effects, including the paralysis observed in mammals after systemic administration of ruthenium red, can be partially accounted for by the binding of ruthenium red to nerve ending membranes, which results in inhibition of Ca^{2+} influx through voltage-sensitive calcium channels and the consequent inhibition of neurotransmitter release (13). In the present study, we ascertained whether ruthenium red affects the 5-HT-induced positive chronotropic effect. We found that the effect of 5-HT was completely inhibited by ruthenium red, while the effects of isoproterenol and histamine were insensitive to ruthenium red. We have reported previously that 5-HT-induced tachycardia observed in the isolated guinea pig atrium is mediated through the activation of 5-HT_3 receptors, which was competitively inhibited by selective 5-HT_3 receptor antagonists (6). Involvement of memantine-sensitive Ca^{2+} entry was suggested in the effect of 5-HT (6). These observations combined with the present results suggest that Ca^{2+} -dependent release of some peptide(s) might be involved in the effects of 5-HT. The peptide(s) released from sensory fibers in the atrium was suggested to express a positive chronotropic effect. On the other hand, peptide release was not involved in the

effects of isoproterenol and histamine, which promote the positive chronotropic effect through specific receptor-mediated activation of adenylate cyclase (14). Furthermore, we examined the effects of capsaicin on the rhythmic activity of isolated guinea pig atrium preparations. Capsaicin itself induced a positive chronotropic effect on first application due to the release of bioactive peptides from their storage sites in sensory fibers (15). On the other hand, the second application of capsaicin did not show any effect on the atrium, indicating complete depletion of bioactive peptides from storage sites and/or desensitization of the release reaction by capsaicin. 5-HT-induced tachycardia was reduced in the capsaicin-pre-treated atrium (after capsaicin) as well as in the presence of capsaicin (with capsaicin). The effects induced by isoproterenol and histamine were not affected by such capsaicin treatment. These observations suggested that 5-HT-induced tachycardia is selectively sensitive to capsaicin treatment and that the effect might be mediated through release of cardiogenic peptide(s). Furthermore, we found that 5-HT-induced tachycardia was potentiated by thiorphan, an inhibitor of peptide degradation (16). This result further supported the suggestion that the effect of 5-HT might be mediated through release of bioactive peptide(s).

Immunohistochemical investigations have demonstrated the existence of bioactive peptides such as substance P, CGRP, somatostatin, VIP and neuropeptide Y in the sensory fibers of atrium preparations (17). Among these peptides, substance P, somatostatin, VIP and neuropeptide Y seem to have no positive chronotropic effects. In Langendorff preparations of guinea pig heart, on the other hand, CGRP-immunoreactive substance was confirmed to be released upon 5-HT application, resulting in positive inotropicity (18). In the present study using isolated guinea pig atrium preparations, CGRP (1–37), a full agonist of CGRP₁-like receptor, was found to act selectively as a potent stimulator of chronotropic action. Furthermore, CGRP (8–37), an antagonist of CGRP₁-type receptors, significantly inhibited 5-HT-induced tachycardia under our experimental conditions, while adrenomedullin (22–52), an antagonist of adrenomedullin receptors that was suggested to have some interactions with CGRP (1–37) (19), showed no such effect. These results strongly suggested that the 5-HT-induced positive chronotropic effect can be elicited by release of bioactive peptide, i.e., CGRP, from capsaicin-sensitive pools in a ruthenium red-sensitive manner. The 5-HT-induced tachycardia might merge in a patho-physiological condition such as platelet-mediated thrombosis where sufficient amounts of 5-HT was released from platelet to activate 5-HT₃ receptors on sensory nerve terminals in the atrium.

The observation that tetrodotoxin failed to affect 5-HT-induced tachycardia might exclude the involvement of 5-

hydroxytryptaminergic interneurons in the effect of 5-HT. 5-HT was suggested to activate 5-HT₃ receptors on the sensory nerve terminals. Thus, the activation of 5-HT₃ receptors on the sensory nerve terminals brought about the ruthenium red-sensitive Ca²⁺ influx and resulted in the release of CGRP from capsaicin-sensitive stores. CGRP then activates CGRP₁-type receptors, which are coupled via Gs to the activation of adenylate cyclase and increase the pacemaker current (*I*_f) (14). The *I*_f has been reported to be modulated by ligand-dependent channels (modulated by cAMP) as well as by voltage-gated channels (activation by hyperpolarization) (20).

In conclusion, our findings demonstrated that 5-HT-induced tachycardia observed in the isolated guinea pig atrium is an indirect effect mediated by the release of CGRP from sensory nerve terminals. However, it should be noted that the effect of 5-HT was blocked only partially by capsaicin treatment, while capsaicin-induced tachycardia was completely blocked by capsaicin pre-treatment. It should also be noted that the maximal effect of 5-HT was not more than 50% of that of CGRP (1–37). These findings suggested that activation of 5-HT₃ might induce release of other neuropeptides together with CGRP.

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